Cell Death and Differentiation (2005), 1–3

www.nature.com/cdd

News and Commentary

PS externalization: from corpse clearance to drug delivery

B Fadeel*[,1] and D Xue[2]

[1] Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
[2] Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, USA
* Corresponding author: B Fadeel, Division of Molecular Toxicology, Institute of Environmental Medicine, Nobels väg 13, Karolinska Institutet, 171 77 Stockholm, Sweden. Tel: +46-8-524-877-37; Fax: +46-8-33-73-27; E-mail: bengt.fadeel@ki.se

Cell Death and Differentiation advance online publication, 9 December 2005; doi:10.1038/sj.cdd.4401836

Phosphatidylserine (PS) externalization on the plasma membrane of aging red blood cells and on apoptotic cell corpses serves as a common recognition signal for macrophages.[1] Perturbation of phospholipid asymmetry and rapid redistribution of PS also occurs in platelets and provides a procoagulant surface that promotes blood clotting.[2] In addition, several recent studies have revealed novel roles for altered plasma membrane phospholipid distribution in nonapoptotic cells. The aim of the present discourse is to highlight these emerging roles for PS externalization, and some of the putative therapeutic implications thereof.

PS signaling is required for programmed cell clearance

Phospholipid asymmetry is a common feature of all eukaryotic membranes. Moreover, mitotic and quiescent cells invest considerable amounts of energy to generate and maintain membrane lipid sidedness. Loss of phospholipid asymmetry and the concomitant externalization of PS can be readily monitored by the use of fluorescence-conjugated annexin V, an endogenous protein that specifically binds to PS, and this process has been well studied in platelets. Thus, when platelets are activated by agonists, such as thrombin and collagen, PS is rapidly brought to the surface of the cell. Exposed PS then catalyzes the assembly of proteins of the coagulation system, leading to a conversion of soluble fibrinogen into an insoluble fibrin clot.[2] The pivotal role of PS externalization is illustrated in Scott syndrome, a bleeding disorder characterized by an impaired Ca^{2+}-induced phospholipid scrambling, and hence an inability of platelets to promote blood coagulation.[2] Apoptotic cells are also procoagulant, and annexin V can completely abolish the procoagulant activity, suggesting that this effect is PS-dependent.[3] More recent findings suggest that annexin V-induced internalization of tissue factor (TF), the initiator of blood coagulation, may also contribute to the anticoagulant functions of annexin V (discussed below).

The ability of PS to act as a signal for *in vivo* recognition and clearance of effete cells was demonstrated 20 years ago in studies of red blood cells.[4] Subsequent studies by Fadok *et al*.[5] showed that PS externalization also can trigger specific recognition and removal of apoptotic cells, and the exposition of PS was soon found to be a common, if not universal, event during apoptosis.[6] More recent studies have shown that PS externalization is, in fact, essential for corpse clearance by macrophages and nonprofessional phagocytes (fibroblasts).[1] Moreover, our studies have indicated that the generation of oxidized PS species (PS-OX) is an integral part of the apoptosis program.[7] Externalization of PS-OX on the cell surface potentiates macrophage engulfment of apoptotic cells, and could also serve to promote the binding of so-called bridging molecules (serum proteins) to apoptotic cell corpses.[8] Interestingly, deletion in mice of the PS-binding bridging molecule, milk fat globule epidermal growth factor 8 (MFG-E8) (also known as lactadherin) results in impaired engulfment of apoptotic cells and autoimmune disease,[9] thus highlighting the physiological importance of PS-mediated corpse engulfment. PS externalization during apoptosis is known to be caspase-dependent,[10] while nonapoptotic PS externalization, on the other hand, may occur in a caspase-independent manner. Moreover, recent studies suggest that the modulation of PS distribution transpires downstream of mitochondrial alterations during apoptosis.[1] Of note, there are differences between the regulation of PS exposure in activated (i.e. nonapoptotic) cells and in apoptotic cells. For instance, apoptosis-induced PS exposure occurs over a much longer time scale (hours) than the Ca^{2+}-induced scrambling process in platelets (minutes).[11]

Contortions of the plasma membrane, commonly referred to as blebbing, are a characteristic feature of apoptosis, and occur downstream of caspase-dependent activation of the ROCK kinase.[1] PS externalization can, in some cases, be divorced from membrane blebbing; however, macrophage engulfment of PS-positive cell corpses appears to be less efficient when blebbing is prevented (unpublished findings). An interesting twist to the story was added when Tepper *et al*.[12] reported that blebbing during apoptosis is related to sphingomyelin (SM) hydrolysis to ceramide and the loss of phospholipid asymmetry in the plasma membrane. Hence, ceramide production during the execution phase of apoptosis was shown to be derived from SM initially located in the outer leaflet of the plasma membrane, which gains access to a cytosolic SMase by flipping to the inner leaflet in a process of lipid scrambling. Alterations of plasma membrane phospholipid distribution may thus have important ramifications, not only for programmed cell clearance, but also during the execution phase of apoptosis. Adding further complexity to this story is the recent observation, reported in the present journal, of the simultaneous cell surface externalization of

protein kinase C (PKC) and PS during enteropathogenic *Escherichia coli* infection.[13] Interestingly, externalized PKC remained biologically active. Moreover, pathogen-triggered externalization of PKC inhibited the full morphological expression of apoptosis, including plasma membrane blebbing. Together, it appears that PS redistribution can promote enzymatic activities inside the cell (SMase-mediated hydrolysis) as well as on the cell surface (PKC-dependent phosphorylation) during the process of apoptosis. Future studies will reveal whether other cell death-related signaling functions also are affected, directly or indirectly, by the externalization of PS.

Emerging roles for PS signaling in nonapoptotic cells

The process of PS externalization has been appropriated for various physiological purposes, and also occurs in nonapoptotic cells. For instance, immunoglobulin E-dependent stimulation of mast cells results in reversible PS externalization in the absence of other features of apoptosis.[14] Furthermore, developing myotubes, as well as megakaryocytes and megakaryoblasts, exhibit PS externalization, and this alteration of the plasma membrane was proposed to be involved in the process of membrane fusion, a critical feature of both myotube formation and platelet release from progenitor cells.[11] A large proportion of nonapoptotic B cells in mice were also reported to bind annexin V, and PS externalization in this cell population was suggested to play a role in antigen receptor-mediated signaling.[15] Moreover, transient, caspase-independent PS externalization occurs on the surface of numerous cell types upon *Chlamydia trachomatis* and *Chlamydia pneumoniae* infection.[16] Of note, a corresponding increase in macrophage engulfment of infected host cells was seen, despite the absence of other signs of apoptosis, suggesting that the only change a cell needs to undergo to be functionally dead (or buried alive) is the exposition of recognition signals for macrophages.[17]

Elliott *et al*.[18] have recently provided evidence for a number of novel signaling roles for PS externalization in nonapoptotic cells. Using a continuous flow cytometric method to detect PS translocation in real time, these investigators showed that PS is constitutively exposed on murine $CD4^+$ T cells which express low levels of the tyrosine phosphatase, CD45. Brief stimulation through the ATP-gated ion channel, $P2X_7$ results in Ca^{2+}-dependent, nonapoptotic PS externalization within seconds,[19] and Elliott *et al*.[18] showed that activation of $P2X_7$ causes changes in PS distribution that modulate signal transduction in several ways. For instance, opening of the $P2X_7$ cation channel itself was shown to require PS redistribution. Furthermore, $P2X_7$-mediated PS externalization stimulated shedding of the homing receptor CD62, suggesting a role for PS translocation in T-cell migration. Of considerable interest, $P2X_7$-stimulated changes in PS asymmetry also resulted in a reversal of P-glycoprotein-mediated drug transport,[18] which may have important implications for the reversal of drug resistance in the clinical setting. Since $P2X_7$ expression is largely restricted to immune cells,[18] coadministration of $P2X_7$ agonists could perhaps be used to potentiate the PS-dependent, immune cell-specific accumulation of drugs.

Annexin V associates rapidly with PS-containing membranes in the presence of Ca^{2+}, and then crystallizes as an extended two-dimensional network. Using an impressive array of biophysical methods, Kenis *et al*.[20] have explored the role of the annexin V protein network at the surface of apoptotic and nonapoptotic cells. They showed that annexin V bends the membrane of PS-positive cells, leading to invagination, budding, and endocytic vesicle formation, as well as cytoskeleton-dependent trafficking of these endocytic vesicles. In a subsequent study, Ravassa *et al*.[21] showed that tissue factor (TF) is internalized in PS-expressing cells through this novel pinocytic pathway. Annexin V was thus suggested to act as a regulator of the receptor repertoire through its ability to internalize PS-expressing membrane patches and the receptors embedded within them. Interestingly, annexin V also caused downregulation of TF activity *in vivo* by smooth muscle cells in a mouse model of carotid artery injury.[21] These novel findings could have therapeutic implications insofar as the annexin V-triggered portal on PS-positive cells could be targeted for drug delivery. Indeed, one could envisage that annexin V may promote internalization not only of membrane-bound receptors but also of ligand/receptor complexes, thereby facilitating uptake of therapeutic agents.

PS-positive, nonapoptotic cells are thought to be able to avoid clearance, yet how this occurs remains unresolved. Perhaps a cofactor for engulfment is missing on viable cells (such as annexin I, a PS-binding protein that is recruited from the cytosol to the cell surface during apoptosis).[22] Alternatively, the amount of PS on the surface of living cells may be insufficient to trigger engulfment. Indeed, recent studies have shown that macrophages have a sensitivity threshold for PS that facilitates reliable discrimination of cell corpses.[23] Furthermore, studies reported in this journal have suggested that a restricted lateral mobility of PS in the plasma membrane and, hence, an insufficient density (or clustering) of PS molecules on the cell surface may allow nonapoptotic cells to escape engulfment.[24] Others have speculated that PS molecules on viable cells could somehow be masked, and unavailable for recognition by macrophages.[11] However, masking of externalized PS *in vivo* by administration of a mutant form of MFG-E8 was recently found to trigger autoantibody production in mice,[25] making this an unlikely explanation. On the other hand, it is conceivable that viable cells could express other, dominant signals that discourage engulfment in spite of PS externalization.

Monitoring death in the living

PS externalization has been exploited recently for imaging purposes in the clinic. Indeed, elegant proof-of-principle studies in mice have demonstrated that fluorescently labeled annexin V can be used for real-time *in vivo* visualization of plasma membrane changes in single cardiomyocytes in the injured heart.[26] Furthermore, the recent administration of technetium-99m-labeled annexin V to human cardiac allograft recipients has revealed the clinical feasibility and safety of annexin V imaging for detection of transplant rejection.[27]

Radio-labeled annexin V is now entering clinical trials for the assessment of therapeutic efficacy in cancer patients, the extent and severity of myocardial infarction, and the screening for acute rejection in heart transplant recipients, and could perhaps serve to obviate invasive biopsies in some cases. However, the clinical usefulness of the annexin V-based imaging method depends, of course, on whether apoptotic and nonapoptotic PS externalization can be discriminated with sufficient accuracy.

Post scriptum

PS-dependent mechanisms of corpse clearance have been conserved through evolution. For instance, a PS receptor has been described in mammals[28] as well as in zebrafish[29] and nematodes,[30] and its expression was shown to be important for efficient cell corpse engulfment *in vivo*.[29–32] In contrast, recent evidence from other investigators suggests that the protein encoded by the PSR gene may not be involved in corpse clearance, but seems to be involved in the regulation of macrophage cytokine responses;[33] the discrepancies between the latter studies could be explained, at least in part, by the fact that these groups have investigated different macrophage populations, derived from mice with different genetic backgrounds. Nonetheless, our ongoing studies have provided direct evidence of PS externalization during apoptosis in *Caenorhabditis elegans* (unpublished findings), and PS externalization has also been documented in the slime mould *Dictyostelium discoideum* and in *Saccharomyces cerevisiae*, suggesting that this is a conserved feature of the apoptosis pathway in unicellular and multicellular organisms. Whether the nonapoptotic signaling roles of PS redistribution delineated above are also conserved between species remains to be determined. From a more general point of view, phospholipids (and, in particular, their oxidatively modified counterparts) appear to have assumed several key roles in apoptosis, at least in mammalian cells, including the cardiolipin-dependent release of proapoptotic factors from mitochondria,[34] and the lysophosphatidylcholine-triggered chemoattraction of macrophages to sites of apoptotic lesions.[35] Further studies in the field of oxidative lipidomics of apoptosis are expected to shed more light on the manifold roles of these ubiquitous cellular signaling molecules.

Acknowledgements

This work was supported by the Human Frontier Science Program.

1. Fadeel B (2003) Cell. Mol. Life Sci. 60: 2575–2585
2. Zwaal RFA *et al.* (2005) Cell. Mol. Life Sci. 62: 971–988
3. Casciola-Rosen L *et al.* (1996) Proc. Natl. Acad. Sci. USA 93: 1624–1629
4. Schroit AJ, Madsen JW and Tanaka Y (1985) J. Biol. Chem. 260: 5131–5138
5. Fadok VA *et al.* (1992) J. Immunol. 148: 2207–2216
6. Martin SJ *et al.* (1995) J. Exp. Med. 182: 1545–1556
7. Kagan VE *et al.* (2002) J. Immunol. 169: 487–499
8. Borisenko GG *et al.* (2004) Cell Death Differ. 11: 943–945
9. Hanayama R *et al.* (2004) Science 304: 1147–1150
10. Martin SJ *et al.* (1996) J. Biol. Chem. 271: 28753–28756
11. Schlegel RA and Williamson P (2001) Cell Death Differ. 8: 551–563
12. Tepper AD *et al.* (2000) J. Cell Biol. 150: 155–164
13. Crane JK and Vezina CM (2005) Cell Death Differ. 12: 115–127
14. Martin S *et al.* (2000) Int. Arch. Allergy Immunol. 123: 249–258
15. Dillon SR *et al.* (2000) J. Immunol. 164: 1322–1332
16. Goth SR and Stephens RS (2001) Infect. Immun. 69: 1109–1119
17. Fadeel B, Orrenius S and Pervaiz S (2004) FASEB J. 18: 1–4
18. Elliott JI *et al.* (2005) Nat. Cell Biol. 7: 808–816
19. MacKenzie AB *et al.* (2005) J. Biol. Chem. 280: 33968–33976
20. Kenis H *et al.* (2004) J. Biol. Chem. 279: 52623–52629
21. Ravassa S *et al.* (2005) J. Biol. Chem. 280: 6028–6035
22. Arur S *et al.* (2003) Dev. Cell 4: 587–598
23. Borisenko GG *et al.* (2003) Arch. Biochem. Biophys. 413: 41–52
24. Appelt U *et al.* (2005) Cell Death Differ. 12: 194–196
25. Asano K *et al.* (2004) J. Exp. Med. 200: 459–467
26. Dumont EA *et al.* (2001) Nat. Med. 7: 1352–1355
27. Narula J *et al.* (2001) Nat. Med. 7: 1347–1352
28. Fadok VA *et al.* (2000) Nature 405: 85–90
29. Hong JR *et al.* (2004) Development 131: 5417–5427
30. Wang X *et al.* (2003) Science 302: 1563–1566
31. Li MO *et al.* (2003) Science 302: 1560–1563
32. Kunisaki Y *et al.* (2004) Blood 103: 3362–3364
33. Böse J *et al.* (2004) J. Biol. 3: 15 (doi:10.1186/jbiol10)
34. Kagan VE *et al.* (2005) Nat. Chem. Biol. 1: 223–232
35. Lauber K *et al.* (2003) Cell 113: 717–730